Effect of Dietary Yeast Supplementation on the Diversity of Hindgut Microbial Populations in Two-Year-Old Quarter Horses

Honor's Research Thesis

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Abstract

Dietary yeast supplementation has been reported to influence the gastrointestinal microbiome in multiple animal species, including horses, with varying results. The objective of this study was to evaluate the effect of a dietary live yeast supplement on the diversity of hindgut microbial populations in 2 yr old Quarter Horse mares. Eight Quarter Horse mares (2.0 ± 0.05 yr) were randomly assigned to one of two treatment groups: yeast or control. All mares received a basal diet consisting of 0.5% BW of a 14% CP pelleted concentrate, with water and mixed grass hay *ad libitum* and were housed in outdoor paddocks with access to shelter at all times. Mares in the yeast treatment group were fed a targeted dose of 2 g (4.5×10^9 cfu)/100 kg of BW per day of a live culture of *Saccharomyces cerevisiae* for 63 d. Fecal samples were collected weekly and pooled by treatment and week. DNA was extracted from the pooled fecal samples and subjected to PCR-DGGE with universal primers specific to 16S rRNA gene sequences to evaluate changes in bacterial diversity. PCR-DGGE images were analyzed using BioNumerics software to generate dendrogram comparisons based on the position and number of bands. There were no differences in band counts representing total bacteria between the treatment groups at the end of the study; however, the dendrogram indicated differences in the microbial profiles of horses fed the yeast supplement. Further investigation with Principal Coordinate Analysis (PCA) also revealed differences in the microbial profiles due to the dietary yeast supplementation. These findings suggest that dietary yeast supplements may influence microbial diversity in the equine gastrointestinal tract but further research using species specific primers is needed.

Introduction

It is widely recognized that the development and maintenance of microbial populations in the equine hindgut are crucial for the overall health and well-being of the horse (Tuoloma *et al*., 1999; Ward *et al*., 2004; Weese *et al*., 2004; Julliand, 2005; Miyauchi *et al*., 2009; Tanabe *et al*., 2014). Changes in the type of microbes and their activity in the gastrointestinal tract of horses, caused by diet or stress, can induce undesirable effects and cause harm to the host. These undesirable effects may include the colonization of pathogenic bacteria in the hindgut or a decrease in luminal pH leading to illness (Pagan, 1998; de Fombelle *et al*., 2001; Jansen *et al*., 2002; Medina *et al*., 2002; Milinovich *et al*., 2008; Biddle *et al*., 2013). Rapid ingestion of a high starch meal can cause a drastic decrease in cellulolytic bacteria due to proliferation of starch-utilizing bacteria, increased lactic acid production and a drop in luminal pH. The decrease in the concentration of cellulolytic bacteria results in a decrease in fibrolytic activity and favors lactic-acid producing bacteria thus further lowering the luminal pH due to lactic acid production (Kern *et al*., 1973; Bellet, 1982; de Vaux and Julliand, 1992; Julliand, 1996; Julliand *et al*., 2001; Biddle *et al*., 2013). Changes in pH and the increase in lactic acid can irritate the lining of the gut and then affect other bacteria through apoptosis as well as possibly change the permeability of the gut lining to allow toxins to cause harm to the host (Pagan, 1998; Biddle *et al*., 2013). The prophylactic use of beneficial supplements, such as probiotics, prebiotics and synbiotics, to prevent or reduce the severity of gastrointestinal upset has been documented in several species including horses (Kopp-Hoolihan, 2001; Medina *et al*., 2002; Ward *et al*., 2004; Tanabe *et al*., 2014).

Prebiotics and probiotics have been added to equine diets in an effort to re-establish homeostasis after gastrointestinal upset due to physiological or environmental stress (Pellegrini

et al., 1999; Ward *et al*., 2004; Weese *et al*., 2004; Berg *et al*., 2005; Tanabe *et al*., 2014). Yeast has been shown to positively affect the hindgut by reducing changes in pH and lactic acid levels in the large intestine after feeding (Medina *et al*., 2002). The addition of yeast to a horse's diet has also been shown to improve cellulose digestibility and reduce a shift in the proportion of lactic acid-producing to lactic acid utilizing bacteria after the ingestion of a meal (Medina *et al*., 2002; Jouany *et al*., 2008). Although yeast has been shown to influence digestibility, studies evaluating the influence of yeast on microbial diversity in the equine gastrointestinal tract have produced mixed results (Medina *et al*., 2002; Julliand, 2005; Jouany *et al*., 2008; Tanabe *et al*., 2014). The objective of this study was to evaluate the effect of a dietary live yeast supplement on the diversity of hindgut microbial populations in two year old Quarter Horses.

Materials and Methods

Eight Quarter Horse mares (2.0 ± 0.05 yr; 3 geldings, 5 fillies) were randomly assigned to two treatment groups, yeast or control, to evaluate the effect of dietary live yeast supplementation in the gastrointestinal tracts of mares. All horses received a basal diet consisting of 0.5% BW of a 14% CP pelleted concentrate, with water and mixed grass hay *ad libitum* and were housed in outdoor paddocks with access to shelter at all times. Horses in the yeast treatment group received 2.0 g/45.4 kg of body weight per day of a live culture of *Saccharomyces cerevisiae*. Fresh fecal samples were collected from the mares on d 7, 14, 21, 28, 35, 42, 49, and 56. After collection, fecal samples were stored at -20º C until further analysis. One gram of feces from each horse was pooled by treatment and day.

DNA was extracted using a Repeated Bead Beating Plus Column RB++C method (Yu and Morrison, 2004) with a modified protocol for elution: 30 µL instead of 200 µL of AE. The

subsequent DNA was purified using a Qiagen mini DNA kit (Qiagen Inc.; Valencia, CA). The protocol from the Q33130 kit was used with the following modifications: 50 µL of working solution was used instead of 200 µL, 2.5 µL of standards or DNA samples were used in each well rather than 10 µL. Purified DNA was subjected to electrophoresis on a 1% agarose gel at 80 v for 1 h. Quantification of DNA was determined using Quant-iT PicoGreen (Molecular Probes Inc.; Eugene, OR). DNA was analyzed using PCR-DGGE. The primers used were specific to the V2-V3 region of 16S rDNA of all bacterial species (universal), bacteria belonging to the phylum *Firmicutes,* and bacteria of the *Streptococcus* genera. The genera included in the *Streptococcus* group were *Streptococcus, Enterococcus, Lactococcus, Vagococcus* and *Tetragenococcus*. The reaction mixture for PCR using universal primers (HDA1, HDA2) (50 µL) contained 0.25 µL of each 100 µM primer and Taq polymerase, 50 ng of the DNA template, 5.00 µL of PCR reaction buffer (Invitrogen; Life Technologies Corp., Eugene, OR), 1.02 µL of BSA, and 3.57 µL of 50 mM $MgCl_2$, and 0.408 µL 100 mM dNTP. Distilled water (Life Technologies Corp., Eugene, OR) was added to each reaction for a final total volume of 50 µL. The PCR reaction mixture using the *Streptococcus* primers (LAC3F, LAC2RGC) (50 µL) contained 0.25 µL of each 100 uM primer and Taq polymerase, 50 ng of the DNA template, 5 µL of PCR reaction buffer (Invitrogen; Life Technologies Corp., Eugene, OR), 1.02 µL of BSA, and 3.57 µL of 50 mM $MgCl_2$, and 0.408 µL dNTP.

Before samples were used for DGGE, 3.0 µL of each PCR product was subjected to 1.0 % agarose gel electrophoresis to confirm successful amplification of the V2-V3 region. Then, 8.0 µL aliquots of PCR product were resolved in a 7.5% polyacrylamide gel containing a 40%-60% gradient of denaturants (formamide and urea) for total bacterial products or a 30%-70% gradient for all other PCR products. The DGGE gel was run in 1.0 % Tris-acetate-EDTA (TAE) buffer at

60° C and 82 v for 16 h using INGENY phorU-2 (Ingeny; Leiden, The Netherlands). The subsequent images were captured using an AlphaImager HP® (ProteinSimple; Santa Clara, CA).

Captured images were uploaded into BioNumerics (Applied Maths NV; Sint-Martens, Latem, Belgium) and analyzed for banding patterns and positions to depict diversity in the form of a dendrogram. The program used a band-searching algorithm to detect bands in the DGGE gels and bands were manually selected. The lanes were converted to densitometric curves and normalized using bands on the standard sample to make sure that the location of bands was consistent across all gels. Clusters were determined by comparing patterns and a dendrogram was used to show relative similarities which is shown by the length of the lines.

Similarity coefficients were derived by comparing pooled samples DGGE profiles (banding patterns, fingerprints) according to time point in BioNumerics. The fingerprints of each of the pooled samples were then depicted with Principal Coordinate Analysis (PCA) in BioNumerics, which evaluated the similarity and dis-similarity of the bacterial profiles and assigned each sample a single point in space. This analysis is used to show the samples relation to each other in space and compare within and between treatments without complex banding patterns or dendrograms.

Bands for each sample were counted after positions were analyzed in BioNumerics. Band counts were compared by treatment group and then analyzed using PROC LOESS in SAS v. 9.3 (SAS Institute; Cary, NC.).

Results and Discussion

No adverse effects due to yeast supplementation were observed during the study. DNA was successfully extracted from all pooled fecal samples (n = 18) and PCR products specific to

each bacterial primer set were successfully obtained using the extracted DNA for all pooled samples. An appropriate PCR product (amplicon) was obtained for the live yeast supplement with the V2-V3 specific primers and was subsequently used as a positive control for the PCR reactions with the fecal samples.

Overall, DGGE band counts remained relatively constant for horses supplemented with yeast in this study whereas DGGE band counts of horses in the control group increased throughout the study (Figure 1). This finding suggests that the hindgut microbial populations remained relatively unchanged in horses fed the yeast supplement and is supported by previous research in which the addition of yeast to a horse's diet was reported to stabilize the microbiota by improving cellulose digestibility and reducing the proportion of lactic acid-producing to lactic acid-utilizing bacteria (Jouany *et al*., 2008; Medina *et al*., 2002).

Interestingly, horses fed the yeast supplement in the present study exhibited an increased number of DGGE bands on d 0, 7 and 14 compared to horses in the control group indicating differences in hindgut microbial populations between the treatment groups at the beginning of the study ($p < 0.05$); however, there were no differences in DGGE band counts throughout the remainder of the study. The difference in these band counts may be due to individual variation between horses' microbial profiles (Biddle *et al*., 2013). Prior studies have also reported large variation in intestinal ecosystems between individual horses, especially when horses are fed high-starch diets (Medina *et al*., 2002). This may have contributed to the significant variation in the present study as concentrates comprised a large portion of their daily feed intake. Diets containing concentrates contain a significantly higher ratio of starch compared to forage only diets. Therefore, horses receiving a diet including concentrates may have increased variation in their microbial populations. Another consideration may be the administration of medications

such as anthelmintics. It was possible that some of the horses may have been given anthelmintics within 60 days prior to the start of the study which may have influenced microbial diversity at the beginning of the study.

The dendrogram and Principle Coordinate Analysis (PCA) showed clustering by treatment group (figure 2). The clusters of mares in the treatment group were more closely related than those in the control group as shown by shorter connecting branches in the dendrogram and less space between points in PCA, especially for d 14, 28, 35, 42, and 56 in the yeast supplemented group (figure 3). Furthermore, previous studies have shown that yeast supplementation has a buffering effect that stabilizes the microbiome which further explains the similarity in band numbers in my yeast supplemented group (Medina *et al*., 2002).

While there was no significant difference between the treatment groups at the end of the study, the clustering of treatment group shown in the dendrogram and PCA indicates that the addition of live yeast to the mare's diet appeared to reduce the variation of overall bacteria in the hindgut of Quarter Horse mares. This supports prior research that reported differences in the microbial profiles of horses fed a yeast supplement compared to controls (Jouany *et al*., 2008; Medina *et al*., 2002). While my study and prior studies suggest a positive outcome from the addition of a prophylactic yeast supplementation, further studies need to be done in order to determine effective dosage.

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Appendix

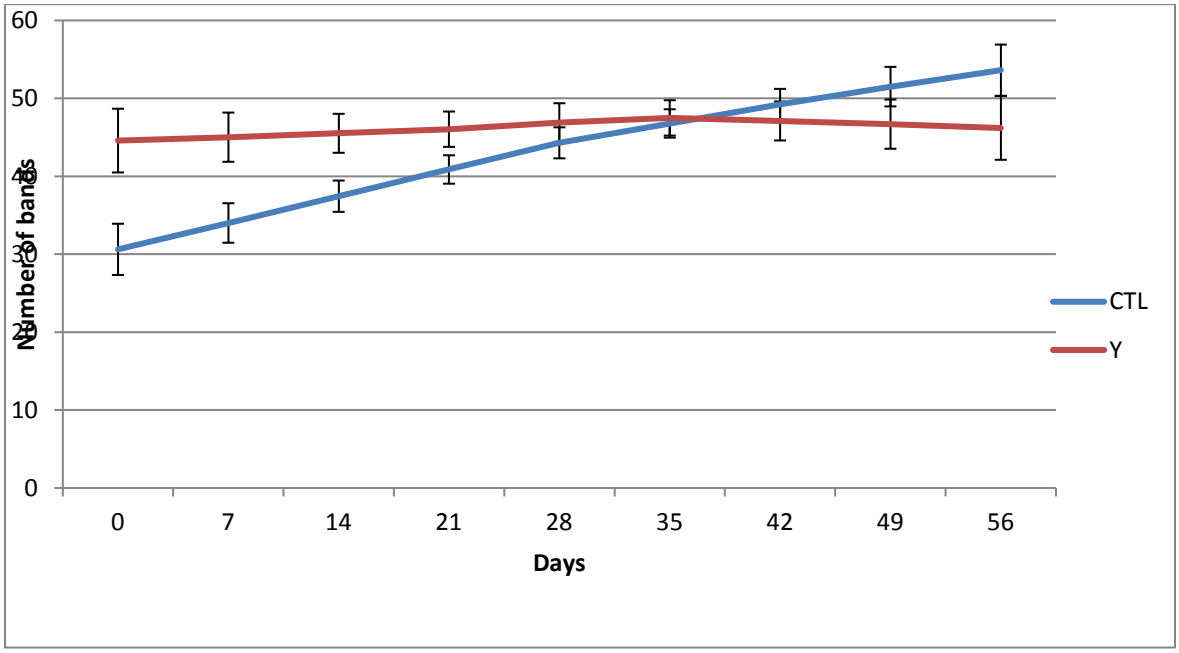

Figure 1. Influence of probiotic supplementation on hindgut microbial diversity in two year-old Quarter Horses (n=8) as shown through band counts. Band counts were generated using primers specific to 16S rRNA sequences. Band counts were analyzed with PROC LOESS of SAS v. 9.3. Significant differences were declared at $P \leq 0.05$ (*).

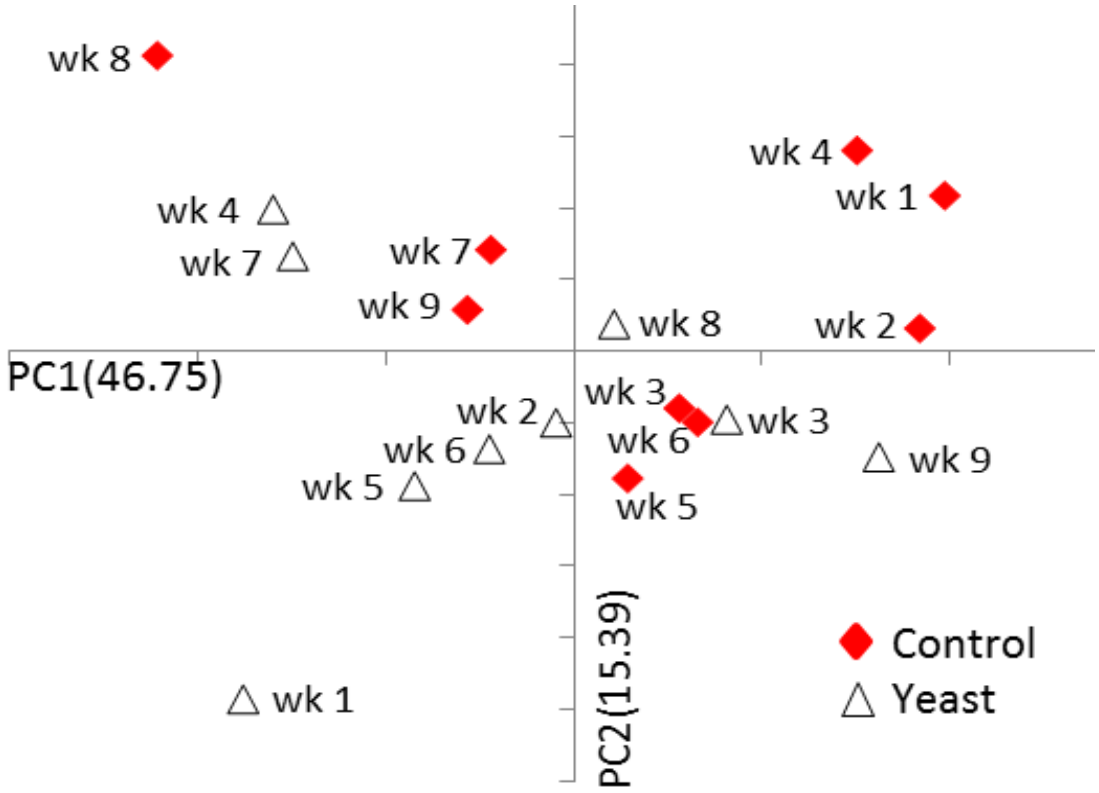

Figure 2. Relatedness of PCR-DGGE profiles representing total bacteria from pooled fecal samples from two year-old Quarter Horses mares (n=8). PCA was generated from a binary similarity matrix to assess the similarity between treatments.

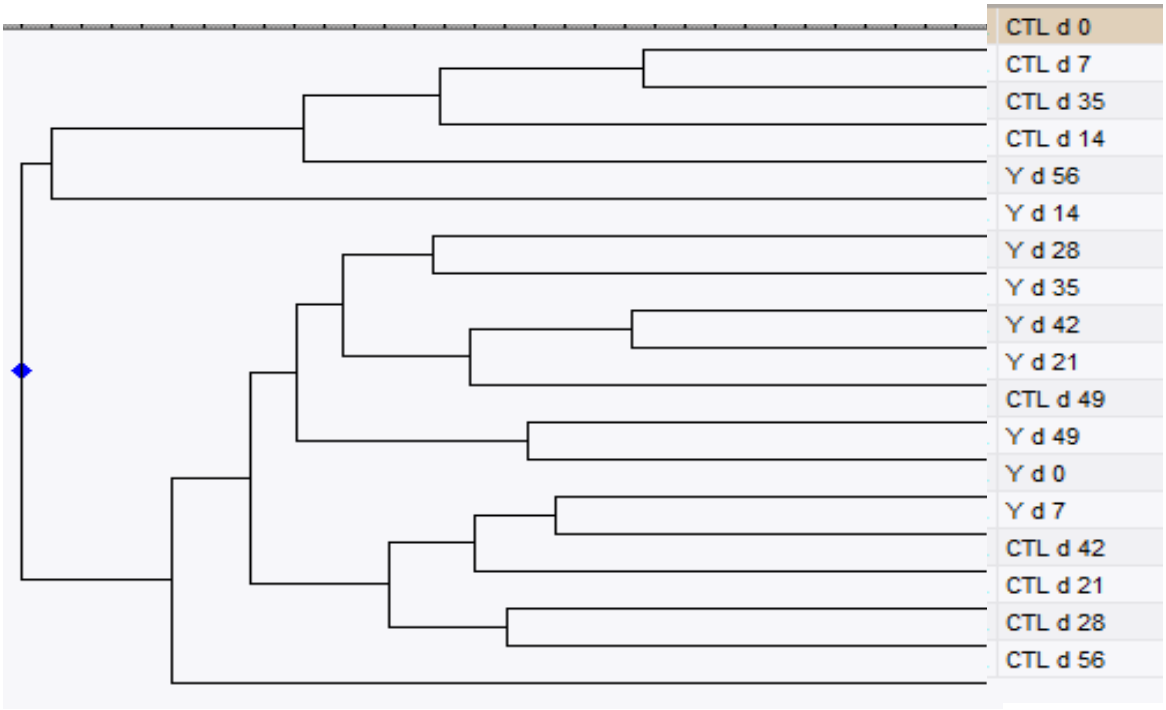

Figure 2. Relatedness of PCR-DGGE profiles representing total bacteria from pooled fecal samples from two year-old Quarter Horses mares (n=8). Dendrograms were generated based on a distance matrix calculated by the Jaccard and unweighted pair-group method with arithmetic average (UPGMA) functions.